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Genotypes of Human Polyomaviruses in Urine Samples of Pregnant Women in Taiwan

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The viral DNA of human polyomaviruses JC virus (JCV) and BK virus (BKV) was detected by the polymerase chain reaction (PCR) in urine samples from 31 pregnant women in Taiwan. A pair of appropriate primers amplified both JCV and BKV DNA of the regulatory region simultaneously in PCR. An oligonucleotide probe homologous to both JCV and BKV regulatory region was used subsequently to detect the viral DNA by Southern blotting after PCR amplification. Approximately 36% of the examined urine samples were human polyomavirus positive. The genotypes of JCV and BKV were determined by DNA sequencing of their regulatory regions. Besides CY archetype, a new strain (Taiwan-1) of JCV with a pentanucleotide (GGGAA) deletion and a new strain (Taichung-1) of BKV with two nucleotide alterations within the regulatory region were found in the urine samples. Eight of the examined samples were JCV infected, one was BKV infected, and two were JCV and BKV mix-infected. The JCV positive individuals were infected by CY archetype and Taiwan-1 strain equally. However, Taichung-1 strain was the only BKV strain found in the BKV positive individuals.

KEY WORDS: papovaviruses; JC virus; BK virus.

INTRODUCTION

The human polyomaviruses, JC virus (JCV) and BK virus (BKV), are spread widely in the human population [Walker and Frisque, 1986]. JCV may cause progressive multifocal leukoencephalopathy (PML) [Padgett and Walker, 1973; Padgett et al., 1976, 1977], a fatal demyelinating disease. The viruses may persist in the kidney in a form called archetype [Yogo et al., 1990]. During an immunocompromised state, the viruses may be reactivated and shed in the urine [Flaegstad et al., 1991; Markowitz et al., 1991; Yogo et al., 1991]. During prolonged immune suppression, new strains of JCV may be formed from archetype by deletions and duplications within the promoter-enhancer regulatory region of the virus, which may be active in brain cells causing PML [Yogo et al., 1991; White et al., 1992]. BKV may be associated with hemorrhagic cystitis [Arthur et al., 1986; Apperley et al., 1987]. In addition, BKV DNA has been found in human pancreatic tumors [Corallin et al., 1987], brain tumors [Dorries et al., 1987], and other neoplastic tissues [Knepper and diMayorca, 1987].

Many epidemiological studies of human polyomaviruses have been carried out. Serological studies have shown some 70% of the adult population to be positive to these viruses [Padgett and Walker, 1973; Brown et al., 1975; Taguchi et al., 1982; Chesters et al., 1983]. The infectivity and genotypes of human polyomaviruses in different areas and different population groups have been investigated extensively [Arthur et al., 1989; Kitamura et al., 1990; Markowitz et al., 1991; Kitamura et al., 1994] to determine the relationship between the virus and pathogenesis.

The prevalence of human polyomavirus infection in the Taiwan area has not been reported. In the current study, the infectivity and genotypes of human polyomavirus was studied in pregnant women in Taiwan. It was found that 36% of the women examined were human polyomavirus positive, including JCV and BKV. In addition, Taiwan-1 strain and CY archetype [Yogo et al., 1991] of JCV and Taichung-1 strain of BKV were found in pregnant women.

MATERIALS AND METHODS

Sample Sources

Urine samples were collected from healthy pregnant women during the second and third trimesters. Sixteen samples, code numbers A1–A16, were collected at the hospital of Chung Shan Medical and Dental College, Taichung, and 15 samples, code numbers A17–A31,

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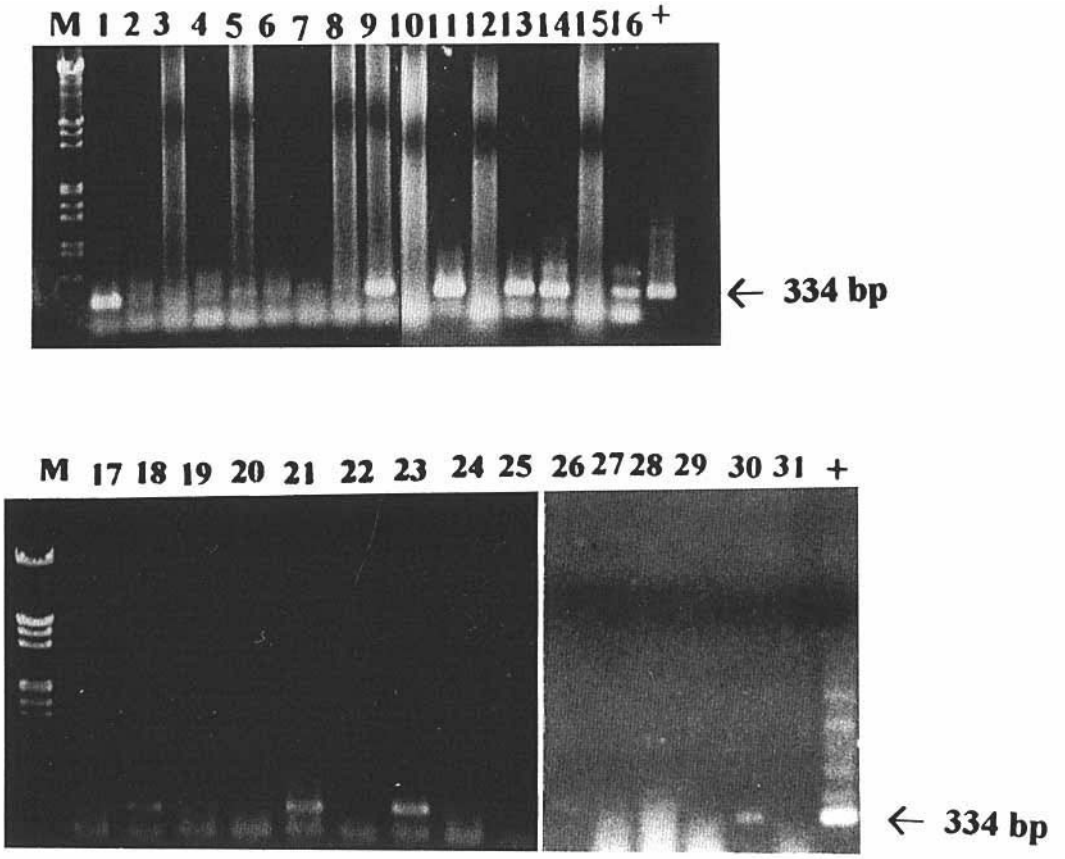

Fig. 1. Electrophoresis of PCR products from urine samples of pregnant individuals. The DNA fragments were amplified by using JB1 primer (5′-CCTCCACACCCTTACTTGAG-3′) and JB2 primer (5′-GTGACAGCTGGCGAACCATGGC-3′), flanking the promoter regions (334 bp) of JCV and BKV. After PCR, the products were separated by 1% agarose electrophoresis and stained by ethidium bromide. **Lane M:** Lambda DNA digested with EcoRI and Hind III; **lanes 1–31:** patients' code number A1–A31; **lane +:** 10 ng of JCV positive control DNA (pM1TCR1A) [Frisque et al., 1984].

were collected at the hospital of National Taiwan University, Taipei. All samples were frozen immediately at −20°C after collection until tested.

Sample Preparation

Urine (10 ml) was ultracentrifuged at 142,000 × g for 90 min at 4°C using a Beckman SW 41 Ti rotor in a Backman L8-70M ultracentrifuge. The sedimented pellet was resuspended in 1 ml of distilled water by brief vortexing. The fluid (5 μl) was mixed with 4 μl of water and 1 μl of 10 × lysis buffer containing proteinase K (100 mM Tris-HCl, 10 mM EDTA, pH 8.0, 500 μg/ml proteinase K). The mixture was incubated at 50°C for 15 min and then at 95°C for 10 min and centrifuged at 10,000 rpm for 3 min. The supernatant was collected and used for the polymerase chain reaction.

Polymerase Chain Reaction

The supernatant from the lysed mixture was used for viral DNA amplification in a reaction mixture containing 9 μl of the lysate supernatant and 41 μl of the polymerase chain reaction (PCR) ingredients. The PCR reagents contained 20 pmole of each primer, JBR1 (5′-CCTCCACGCCCTTACTACTTCTGAG-3′) and JBR2 (5′-GTGACAGCTGGCGAAGAACCATGGC-3′ [White et al., 1992], 5 μl of 10 × Taq DNA polymerase buffer, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, and two units of Taq DNA polymerase (Promega, WI). The PCR reaction mix was overlaid with 50 μl of mineral oil (Sigma, St. Louis, MO). After a 30-sec centrifugation at 10,000 rpm, the PCR reaction was followed. The initial step of PCR was 95°C for 3 min, and then 40 cycles of 95°C for 45 sec (denaturation), 55°C for 2 min (annealing), and 72°C extension for 2 min were followed by 72°C for 4 min to extend the last cycle using a Perkin-Elmer Cetus DNA thermal cycler.

PCR Products Analysis by Electrophoresis

Five μl of the PCR reaction mix of each sample were loaded into a 1.5% agarose (molecular-biology grade; IBI Biotechnologies, New Haven, CT) gel and subjected to electrophoresis in TBE buffer (90 mM Tris-HCl, pH 8.3, 90 mM sodium borate, 2.5 mM EDTA) at 80 volts for 30 min. The agarose gel was stained with ethidium bromide (0.5 μg/ml) for 5 min and then photographed under UV light.

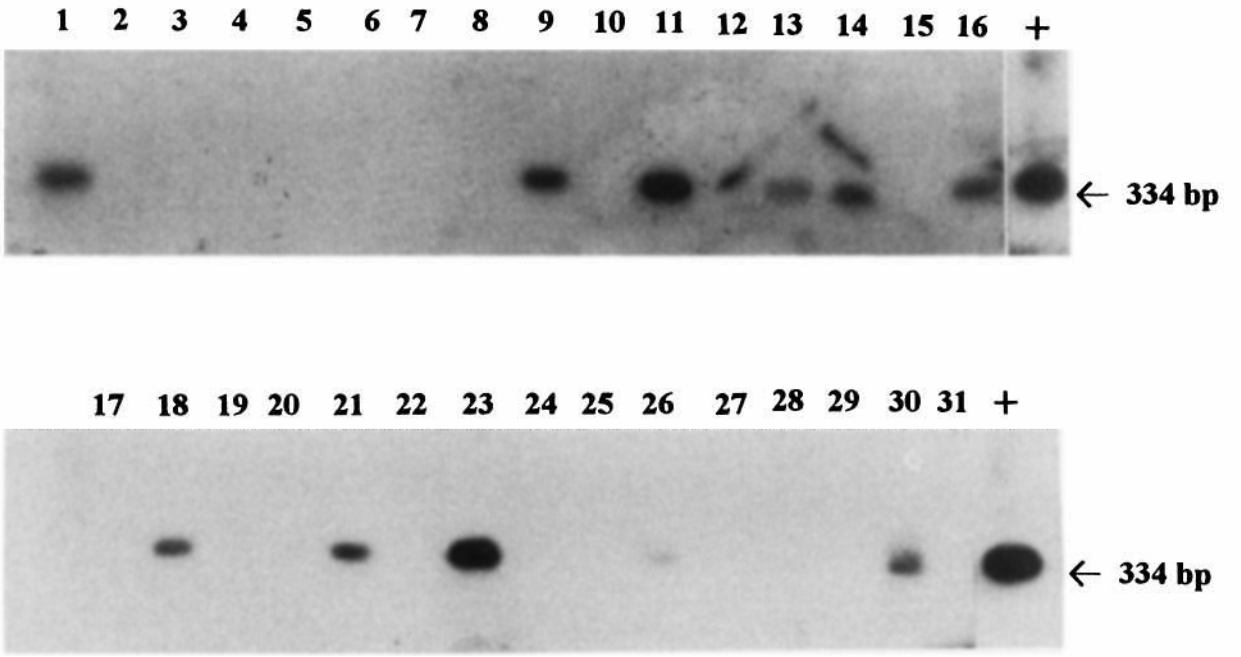

Fig. 2. Southern blot of JCV and BKV regulatory region. The DNA fragments of PCR products were electrotransferred onto nylon membrane after electrophoresis, probed with JCV and BKV specific ^{32}P-labeled oligonucleotide 5′-CCTAGGGAGCCAACCAGCTAACAGC-3′, and visualized by autoradiography. **Lane M:** Lambda DNA digested with EcoRI and Hind III; **lanes 1–31:** patients' code numbers A1–A31; **lane +:** 10 ng of JCV positive control DNA (pM1TCR1A) [Frisque et al., 1984].

Southern Blot of PCR Product

PCR reaction mixture (10 μl) was electrophoresed in a 1.5% agarose gel in TAE buffer (40 mM Tris-acetate, pH 8.5, 2 mM EDTA). For Southern blot, the DNA fragments in the agarose gel were transferred onto a nylon membrane (MSI, Westboro, MA). The membrane was treated initially with an alkaline solution (0.2 N NaOH and 0.6 M NaCl) for 1 hr and then treated with Tris buffer (1.0 M Tris, pH 7.4, and 0.6 M NaCl) for 1 hr. Prehybridization was followed by treating the membrane with 5 × Denhardt's solution, 20 μg each of bovine serum albumin (Sigma), Ficoll (Pharmacia, Piscataway, NJ), and Polyvinylpyrrolidone (Sigma) per ml), and 6 × SSC (0.9 M NaCl and 0.09 M sodium citrate) at 58°C for 5 hr. The solution was replaced with a fresh hybridization solution of 5 × Denhardt's, 6 × SSC, and 0.5% sodium dodecyl sulfate (SDS) containing 4×10^5 cpm/ml of (^{32}P) ATP-end-labeled JBR-3 oligonucleotide probe (5′-TGGCAGTTATAGTGAAACCCC-3′). Following 5 hr of hybridization at 58°C with shaking, the nylon membrane was washed five times with 2 × SSC solution with 1.0% SDS at 58°C for 3 min each, five times with 1 × SSC solution with 1.0% SDS at 58°C for 3 min each wash. The membrane was air dried and exposed on Kodak X-AR film for overnight at −70°C.

DNA Sequencing

Thirty μl of the PCR reaction mixture of the JCV or BKV positive samples were loaded on a 1.5% low melting agarose (Promega) gel in TAE buffer for electrophoresis. The band containing the DNA fragment was visualized by staining the gel with ethidium bromide and excised under UV light. The DNA fragment in the low melting agarose was purified by Magic PCR Minipreps kit (Promega). The purified DNA fragment was sequenced directly by using the *f*mol DNA sequencing kit (purchased from Promega, which also provided the protocols).

RESULTS

Viral DNA Amplification by PCR

Thirty-one urine samples were collected from healthy pregnant women. Following ultracentrifugation, the urine pellets were treated with lysis buffer to release viral DNA. The released viral DNA in the solution was used as template for PCR amplification directly. For PCR amplification, primers JBR1 and JBR2 were employed to detect JCV and BKV DNA simultaneously, because the primers anneal to the constant ends of regulatory regions (nucleotide numbers −45–−21 and 265–289) of both JCV and BKV [Markowitz et al., 1991]. After PCR, the reaction mixtures were analyzed by agarose electrophoresis. The results showed that 11 of 31 urine samples contained a 334 base-pair DNA fragment (Fig. 1, lanes 1, 9, 11, 13, 14, 16, 18, 21, 23, 26, 30). The size of the DNA fragment generated by JBR1 and JBR2 primers was expected to be 334 base pairs as shown in the control lane (Fig. 1, lane +). Therefore, the PCR DNA amplification showed that 11 urine samples were presumably human polyomavirus

positive. To rule out possible false-negative results, the negative samples were spiked with 10 pg of DNA of Mad-1 JCV genome cloned into pBR322 [Frisque et al., 1984]. All of the negative samples were found positive after spiking with Mad-1 JCV genome (data not shown).

Southern Blotting

Southern blot analysis was employed to confirm the 334 base-pair DNA fragment of PCR product from urine samples were the regulatory region of human polyomaviruses and to confirm that the negative urine samples did not contain a small amount of DNA fragment that could not be detected by ethidium bromide staining after PCR. JBR-3 oligonucleotide probe, which annealed at nucleotide 140–160 for JCV and 117–138 for BKV regulatory region, was end-labeled with (^{32}P) ATP and hybridized with the nylon membrane containing the PCR reaction mixtures. The results of Southern blot showed that 11 of 31 samples were JCV or/and BKV positive (Fig. 2, lane 1, 9, 11, 13, 14, 16, 18, 21, 23, 26 and 30). The results of Southern Blot were the same as that of agarose electrophoresis with ethidium bromide staining shown in Fig. 1. These results confirmed that the 334 base-pair DNA fragments generated by PCR were the regulatory region of human polyomaviruses and the negative samples did not contain a small amount of viral DNA fragments.

DNA Sequencing of the Regulatory Regions

The Southern blot positive samples were subjected subsequently to DNA sequencing to identify the viral strains present in Taiwan and for further molecular characterization of the viruses. The DNA fragments of viral regulatory region of the PCR products were purified from low melting agarose gel and sequenced by *f*mol thermal cycling system.

DNA sequencing revealed that two different genotypes, CY and Taiwan-1, of JCV (Fig. 3) and one archetypal BKV, Taichung-1 (Fig. 4), were found in the human polyomaviruses positive urine samples. Five samples including A1, A13, A14, A18, and A21 were the CY archetype of JCV. In addition, A11, A16, A23, A26, and A30 contained the Taiwan-1 regulatory region of JCV. The difference between CY and Taiwan-1 strain is that Taiwan-1 strain had a pentanucleotide (GGGAA) deletion at nucleotide 218–222 when compared to CY strain [Yogo et al., 1990] (Fig. 3). Three individuals, samples A9, A13, and A14, were BKV infected. The BKV present in the urine samples was Taichung-1 strain exclusively (Fig. 4). Taichung-1 strain had two point alterations at nucleotide 52 (A → T) and 65 (T → C) (Fig. 4) when compared to the WW archetype of BKV [Rubinstein et al., 1987]. Sample A9 was infected by Taichung-1 BKV only, but samples A13 and A14 were mix-infected by both CY JCV and Taichung-1 BKV. Furthermore, based on densitometry of the DNA sequencing films of samples A13 and A14, the ratio of JCV and BKV DNA was ~2 to 1 for both samples. The total infectivity of the human polyomaviruses to the

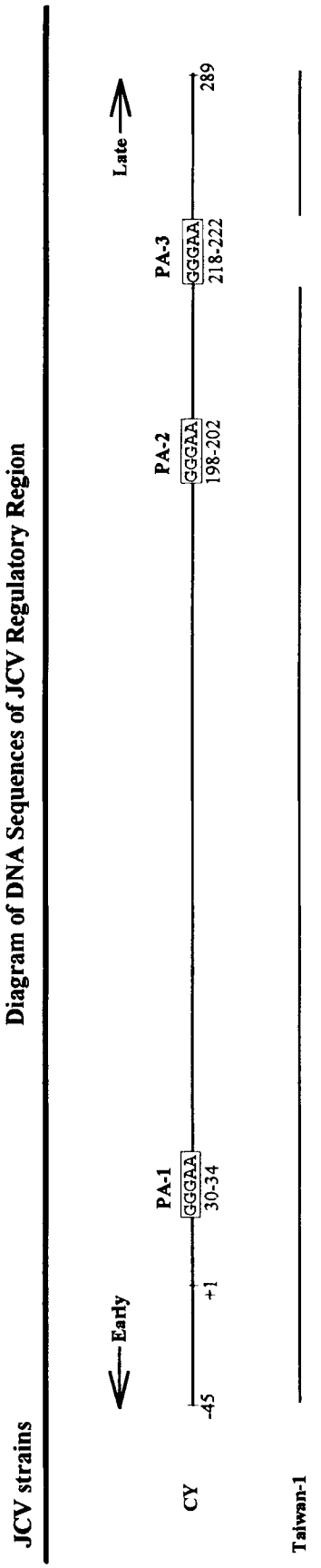

Fig. 3. Comparison of DNA sequences of CY and Taiwan-1 JCV regulatory regions. The DNA sequences of CY and Taiwan-1 JCV regulatory region were sequenced from −45 to 289. CY archetype contains three copies of GGGAA pentanucleotide (PA-1, PA-2, and PA-3), but Taiwan-1 strain has only two copies of GGGAA (PA-1 and PA-2).

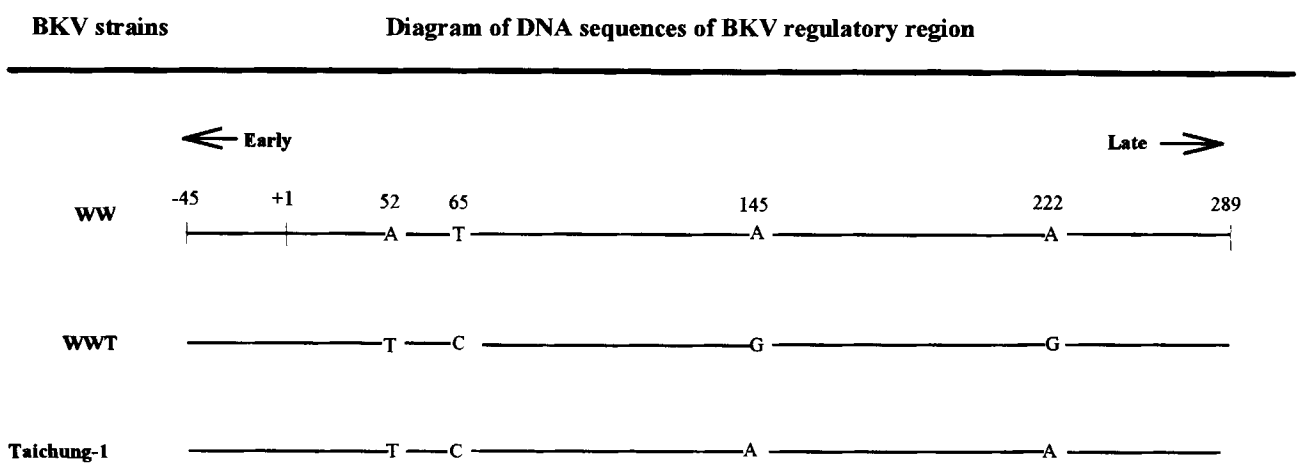

Fig. 4. Comparison of DNA sequences of WW, WWT, and Taichung-1 BKV regulatory regions. The DNA sequences of Taichung-1 BKV regulatory region was sequenced form -45 to 289. Nucleotides 52, 65, 145, and 222 are various among WW [Rubinstein et al., 1987], WWT [Sundsfjord et al., 1990], and Taichung-1 BKV.

examined samples were ~36%: 25.8% of these samples were infected with JCV, 3.2% were infected with BKV, 6.5% was infected with a mixture of JCV and BKV (Table I).

DISCUSSION

Although it has been shown that >70% of the adult population are antibody positive to the human polyomaviruses [Padgett and Walker, 1973; Brown et al., 1975; Taguchi et al., 1982; Chesters et al., 1983], little is known about the proportion of carriers in different geographic populations. The viruses can be reactivated during immunocompromised states and are shed in the urine [Coleman et al., 1980; Shah et al., 1980; Gibson et al., 1981]. Pregnancy is one of the conditions that may cause viral reactivation. Therefore, the urine samples of pregnant women in Taiwan were examined for the presence of human polyomaviruses. In this study, it was found that the CY archetypal strain [Yogo et al., 1990], the Taiwan-1 strain of JCV, and the Taichung-1 strain of BKV were present in Taiwan. About 25.8% of the urine samples were infected by JCV, 3.2% were infected by BKV, and 6.5% were infected by both JCV (CY strain) and BKV (Taichung-1 strain) (Table I) in this study. Both the CY and Taiwan-1 strains of JCV were found in the urine samples collected from Taipei (north of Taiwan) and Taichung (midwest of Taiwan), but Taichung-1 BKV was found only in the samples collected in Taichung area. Interestingly, Markowitz et al., [1991] found that ~7% of urine samples of pregnant women were JCV positive and 15% were BKV positive. Those urine samples were collected in Denver, CO. Kitamura et al. [1990] have shown that the urine samples of older individuals in the Tokyo and Chiba areas of Japan were infected by BKV and JCV at ~9% and 52% respectively. About 7% of healthy adults were shedding both JCV and BKV in urine [Arthur et al., 1989]. More recently, Kitamura et al. [1994] have shown that 45.7% of the age group between 20 to 29 were infected by JCV in the Tokyo area. The prevalence of the human polyomaviruses is apparently worldwide, although the epidemiological distribution of BKV and JCV is variable.

The CY archetypal strain has been isolated from urine samples of nonimmunosuppressed individuals [Yogo et al., 1990]. The BKV and JCV shed in urine of pregnant women are predominately archetypal strain [Markowitz et al., 1991]. More recently, White et al. [1992] showed that JCV present in kidney tissue of non-PML patients was archetype, but the JCV present in the brain tissue of PML or non-PML patients is "PML-type" variant strains. Based on these investigations, it is possible that JCV may be present in kidney tissue of most healthy individuals with archetypal strain and that long-term immunosuppression may cause genetic rearrangement of viral DNA and lead to infection of brain cells resulting in PML. The JCV strains found in the present study were CY archetype (50%) and Taiwan-1 strain (50%) (Table I). Three copies of GGGAA pentanucleotide are present within the regulatory region and located at nucleotide 30–34, 198–202, and 218–222, respectively (Fig. 3) of the CY archetype [Yogo et al., 1990]. The Taiwan-1 strain had a copy of GGGAA pentanucleotide deletion at the regulatory region nucleotide 218-222. Tada and Khalili [1992] found a brain-derived DNA binding protein LCP-1 (lytic control element-binding protein 1), which was able to bind the lytic control element (AGGGAA GGGA). It has been shown that the lytic control element (LCE) downregulates transcription initiation from the viral late promoter as well as a heterologous promoter in glial cells [Tada et al., 1991]. Taiwan-1 has a copy of GGGAA (a part of LCE) deletion that may be involved in the regulation of transcription. Kumar et al. [1994] have shown recently that GGG regions in the LCE are required for glial cell-specific transcription.

TABLE I. Summary of Human Polyomavirus Genotypes in Pregnant Individuals in Taiwan

Virus	Positive (n = 31)	Genotypes
JCV	8 (25.8%)	Taiwan-1 50% CY 50%
BKV	1 (3.2%)	Taichung-1 100%
JCV + BKV	2 (6.5%)	CY + Taichung-1 100%

However, the importance of the pentanucleotide GGGAA in viral gene regulation has to be examined further.

The BKV found in pregnant women in Taiwan was Taichung-1 strain exclusively (Table I). The nucleotide number 52 and 65 in the regulatory region were T and C for both the Taichung-1 and WWT strains [Sundsfjord et al., 1990], but they were A and T for the WW strain [Rubinstein et al., 1987]. However, these three strains with a few nucleotides alteration and without major deletion or duplication could be grouped as BKV archetypes. Interestingly, the major capsid protein VP1 of Taichung-1 strain has been cloned into a prokaryotic expression vector in our laboratory. The VP1 gene of Taichung-1 was found to have >50 mutations when compared to that of the Dunlop strain [Sief et al., 1979]. It remains to be determined whether the nucleotide alterations on the regulatory and structural regions could affect the tissue tropism and pathogenesis.

This investigation of the genotypes of human polyomaviruses in pregnant women in Taiwan provides significant preliminary information for further studies on the linkages between the viruses and their pathogenesis in Taiwanese population.

ACKNOWLEDGMENTS

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